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Developmental and Pharmacological Studies of Acetylcholinesterase-defective Mutants of *Caenorhabditis elegans*

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ABSTRACT—Three genes (*ace-1, ace-2,* and *ace-3*), which code for acetylcholinesterase (class A, B, and C) have been identified in the nematode *Caenorhabditis elegans*. Here we investigate the developmental and pharmacological properties of mutants retaining only one of distinct classes of acetylcholinesterase. Both class A and B acetylcholinesterases share about one-half of the total enzyme activity throughout development and maximum specific activity at the first larval stage, and are sufficient for maintaining normal acetylcholine levels. Although class B acetylcholinesterase is distributed at the head and the body region as seen in the wild type, the class A enzyme is biased towards the head region. Class C acetylcholinesterase occupies only a few percent of the total activity, is mainly distributed in the body, shows maximum specific activity at the late larval stage and is clearly different in pharmacological response from class A and B acetylcholinesterases. We propose that class B is the major acetylcholinesterase, and that classes A and C are supplementary.

INTRODUCTION

Acetylcholinesterases (AChE) have been extensively studied in various organisms (for reviews, see [5, 13]). It has been proposed that the enzyme has roles additional to the hydrolysis of acetylcholine (ACh), such as the regulation of membrane excitability, permeability, general metabolism or the inactivation of neuropeptides. However, many problems remain to be solved, for example, why the enzyme is present in neurons which has neither choline acetyltransferase, the synthetic enzyme for ACh, nor any identified cholinergic input, and in neurons, which use transmitters other than ACh. These findings raise the question of what role AChE would be playing in noncholinergic cells.

Caenorhabditis elegans is a desirable organism for such studies [15]. Because genetic and biochemical properties of AChE are extensively studied [3, 8, 10]. In *C. elegans,* three genes relating to AChE activity have been identified: *ace-1, ace-2* and *ace-3.* These three classes of AChE (A, B, and C) corresponding to the three *ace-1, ace-2,* and *ace-3* genes have been characterized biochemically, molecularly and genetically [9, 12]. Mutations of any of the three *ace* genes do not result in any visible defects. In the *ace-1 ace-2* double mutant, movement is abnormal and the *ace-1 ace-2 ace-3* triple mutant is lethal at the post-embryonic stage [3, 8, 10]. Therefore, any one of the three *ace* genes is sufficient for survival, and *ace-1* or *ace-2* are necessary for normal movement.

Although much is known about the kinetic properties of the three classes of AChE, the developmental or pharmacological differences among them are not understood. To elucidate the functional role of each class of AChE, we studied the developmental changes in AChE activity in relation to enzyme localization and ACh levels. Several organophosphates inhibit *C. elegans* AChE activity but the correspondence between the extent of the enzyme inhibition and the fatal effect is not always coincident [9]. The relationship between enzyme inhibition and pharmacological action has not yet been systematically studied. An organophosphate, trichlorfon {(2,2,2-trichloro-1-hy-

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droxyethyl)-phosphonic acid dimethyl ester], is a potent nematocide, which probably functions by inhibiting AChE. We studied the response to trichlorfon of mutants defective in two of three *ace* genes. Our experiments indicate that class A and class B AChE have similar properties but class C AChE is greatly different from the two classes AChE in expression, distribution and response to organophosphates. In addition, we describe that class B AChE is indistinguishable from the wild type in distribution and the staining intensity, suggesting that class B is the major AChE of the three.

MATERIALS AND METHODS

Nematode strains. The following genes and mutations were provided from J.Rand (Oklahoma Medical Research Foundation, USA): *ace-1* (*p1000*) X, *ace-2* (*g72*) I, *ace-3* (*dc2*) II, *ace-1* (*p1000*) *ace-2* (*g72*), *ace-2* (*g72*) *ace-3* (*dc2*), *ace-3* (*dc2*) *ace-1* (*p1000*). *C. elegans* was cultured on NGM agar as described by Brenner [2], except that in some cases, a higher concentration of bactopeptone (20 mg/ml) was added to the medium.

Histochemical staining of AChE. AChE in whole mounts of nematodes was stained by the modified method developed by Karnovsky and Roots [11] and by Culotti *et al.* [3]. Nematodes, permeabilized with 95 % acetone and extensively washed, were suspended in a mixture of 10 mg acetylthiocholine iodide, 65 ml 100 mM malate buffer(pH 6.0), 0.5 ml 100 mM sodium citrate, 1 ml 30 mM $CuSO_4$, 1 ml H_2O and 1 ml 5 mM potassium ferrocyanide, and were shaken at 37°C for 30 min. The reaction was stopped by diluting and washing. A DAB-nickel solution(3,5-diaminobenzoic acid 4 mg, ammonium nickel 60 mg, and 10 ml 10 mM Tris(pH 7.6)) containing 0.003% H_2O_2 were added and shaken for 3 min at room temperature to intensify the staining. The DAB-nickel was removed and the slides were washed several times with 10 mM Tris (pH 7.6).

Acetylcholinesterase assays. Organisms were suspended in two volumes of 50 mM Tris pH 7.6 and 0.03% Triton X-100 and homogenized in liquid nitrogen. The homogenates were mixed with class specific assay mixtures [10] as follows. Wild-type AChE was assayed in 95 mM sodium phosphate buffer (pH 8.0), 0.32 mM 5-dithiobis-2-nitrobenzoic acid and 0.47 mM acetylthiocholine iodide. In addition to this assay mixture, 0.13% deoxycholate for assay of class A AChE and 0.03% Triton X-100 for class B AChE, and 0.03% Triton X-100, 0.013% deoxycholate and 2.5 μM neostigmine bromide for class C AChE were added. The final reaction volume was 1585 μl. The reaction was started by adding the homogenate at 25°C and the absorbance at 412 nm was read [4]. Homogenates from the *ace-1 ace-2* mutants were added to a final concentration of 0.3 mg/ml and from the remaining strains, of 0.03 mg/ml.

Acetylcholine assay. Acetylcholine was assayed by the enzymatic conversion of ACh to [^{32}P] choline in the presence of [γ-^{32}P] ATP, acetylcholinesterase and choline kinase [14].

Assay of paralysis and its recovery. Worms were suspended in M9 buffer containing trichlorfon. Paralysis was followed by measuring the frequency of propagating waves along their bodies as described elsewhere [6]. For recovery, completely paralyzed worms were washed with M9 buffer and resuspended in the buffer.

RESULTS

Developmental changes in AChE activity

Changes in the activity of each class AChE with development were compared (Fig. 1). Class A and B AChE activities were maximum at the first larval (L1) stage and the activity decreased gradually with the progress of development. Class C AChE occupied less than 2% of the total AChE throughout development and reached the maximum activity at the later larval stage. Thus, the synthesis of the class C enzyme might be differently regulated from that of the other two.

Developmental changes in AChE distribution

To determine a more detailed developmental pattern of AChE expression, organisms were

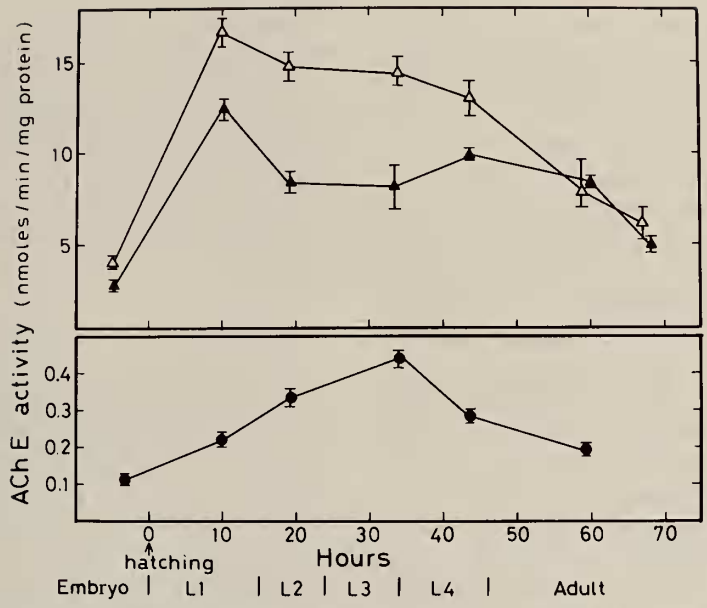


FIG. 1. Changes in AChE activities throughout development. Organisms were homogenized and suspended in the class-specific AChE mixtures [8]. AChE activity was measured at 25°C by the thiocholine method [11]. The embryonic stage is arbitrarily plotted. The mean values of three assays are presented with bar for standard deviation. *ace-2* (*g72*) *ace3* (*dc2*) (▲), *ace-3* (*dc2*) *ace-1* (*p1000*) (△), *ace-1* (*p1000*) *ace-2* (*g72*) (●).

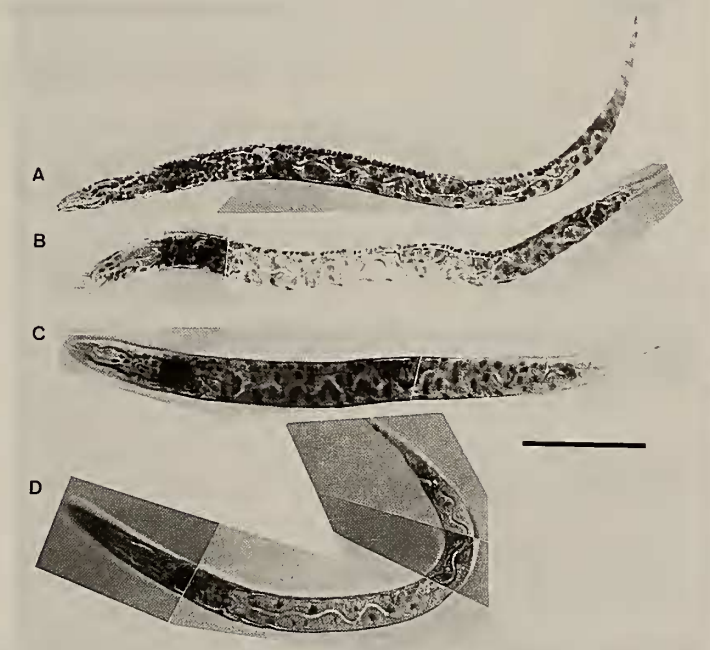


FIG. 2. AChE staining of the second stage larvae. Wild type (A), *ace-2* (*g72*) *ace-3* (*dc2*) (B), *ace-3* (*dc2*) *ace-1* (*p1000*) (C), *ace-1* (*p1000*) *ace-2* (*g72*) (D). Animals are oriented anterior side to the left and the ventral side on top. Bar=100 μm.

stained with acetylthiocholine by the method of Karnovsky and Roots [11]. Culloti *et al.* [3], who pioneered AChE staining with *C. elegans,* found that AChE was abundant in nearly all cells at the early larval stage, then was reduced gradually, but remained mainly in the area of the nerve ring, ventral ganglion, the pharyngeo-intestinal valve and the tail neural region as development progressed. We obtained the same results with the wild-type animal and compared the changes in the staining pattern of the three distinct classes of AChE with the *ace* double mutants from embryo to adult. Representative staining of the second stage larva is shown (Fig. 2). The AChE staining was first detectable on one of two-blastomeres and increased to stable, high levels in all blastomeres at around the 24-cell stage (data not shown). AChE expression in the three double mutants approximately coincided with that of wild type, though the extent of the staining differed. In the newly hatched wild-type larva, nearly all somatic cells and the nerve ring were intensely stained (Fig. 6 A-D). In the *ace-3 ace-1* double mutant, the staining pattern was similar to that of wild type. In the *ace-2 ace-3* double mutant, the head region was stained as in wild type but the body region was less intensely stained. In the *ace-1 ace-2* mutant, the non-neural cells were scarcely stained but the nerve ring and the ventral nerve cord were detectably stained. At the second larval stage in all strains, nonneuronal cells were weakly stained but that of neuronal cells remained intense (Fig. 2). The staining intensity of the positive regions between *ace* mutants was similar to that of the first larval stage.

Developmental changes in ACh levels

At three developmental stages, the ACh levels of three mutants were compared with those of wild-type animals (Fig. 3). The ACh levels in the wild type were high at the larval stage, but decreased to about 25% at the adult stage. ACh levels of the *ace-2 ace-3* and the *ace-3 ace-1* mutants were similar to those of wild-type animals. In the *ace-1 ace-2* mutant, abnormal elevation of ACh was observed at the adult stage, though it was normal at the early larval stage. Therefore, class A and B AChE are sufficient but class C AChE is insufficient for maintaining normal ACh levels.

Inhibition of AChE by trichlorfon

Of the organophosphates tested, trichlorfon was

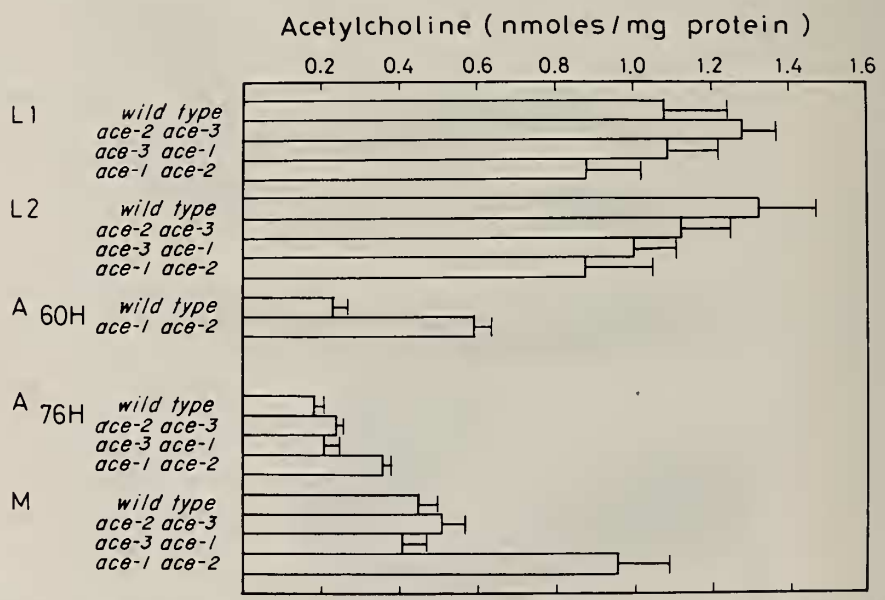


FIG. 3. Changes in ACh levels of wild type and *ace* double mutants. The indicated time at the adult stage means hours after hatching. L1, first stage larva; L2, second stage larva; A, adult; M, asynchronous population. Standard deviations of mean values of three assays are indicated by vertical bars.

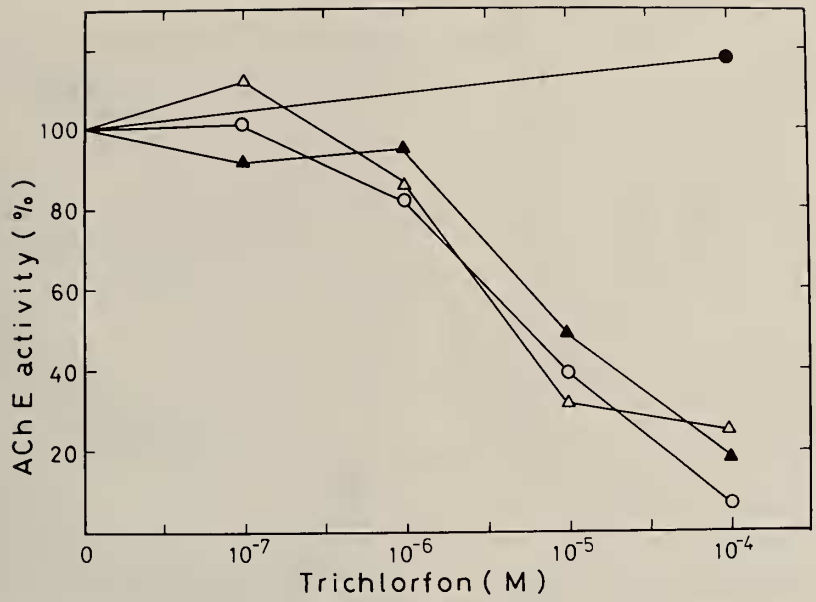


FIG. 4. Inhibition of three classes AChE by trichlorfon. Wild type (○), *ace-2* (*g72*) *ace-3* (*dc2*) (△), *ace-3* (*dc2*) *ace-1* (*p1000*) (▲), and *ace-1* (*p1000*) *ace-2* (*g72*) (●).

the most prominent nematocide [7]. Although several organophosphates have been identified as AChE inhibitors, trichlorfon has not yet been tested [9, 12]. As shown in Figure 4, crude extracts of class A and B AChE were similarly inhibited but class C AChE was not, at the concentrations of trichlorfon tested.

Survival sensitivity of ace *mutants to trichlorfon*

Survival of the *ace* mutants was tested in the presence of trichlorfon (Table 1). Wild-type animals failed to propagate their progeny above 20 μM trichlorfon. Mutation of the *ace-1* or *ace-2* genes did not influence survival. However, mutation of the *ace-3* gene brought about much higher sensitivity to the drug. These results suggest that the trichlorfon sensitivity of wild-type animals is determined by the class C AChE that is encoded by the *ace-3* gene. This hypothesis was tested with mutants preserving one of the three *ace* genes. The sensitivity of the *ace-1 ace-2* double mutant did not differ from that of the wild-type. However, both the *ace-3 ace-1* and the *ace-2 ace-3* mutants were hypersensitive to trichlorfon, supporting the above hypothesis. The *ace-2 ace-3* mutants were slightly but reproducibly more sensitive to trichlorfon than the *ace-3 ace-1* mutants.

TABLE 1. Sensitivity of *ace* mutants to trichlorfon

Mutations	Trichlorfon μM
wild type	20
ace-1 (*p1000*)	20
ace-2 (*g72*)	20
ace-3 (*dc2*)	2
ace-2 (*g72*) *ace-3* (*dc2*)	1
ace-3 (*dc2*) *ace-1* (*p1000*)	2
ace-1 (*p1000*) *ace-2* (*g72*)	40

To determine sensitivity, three fourth stage larvae were put onto NGM agar plates containing various concentrations of trichlorfon (0, 0.4, 1.0, 2.0, 4.0, 10, 20, 40 and 100 μM) and grown at 20°C. The highest concentration of trichlorfon is indicated in which worms, in triple experiments, produced F_2 progeny within ten days.

Paralytic sensitivity of ace *mutants to trichlorfon*

When *C. elegans* was exposed to trichlorfon, the animals gradually became immovable. The pattern of trichlorfon-induced paralysis differed in each *ace* double mutant (Fig. 5). Animals retaining only the *ace-3* genes were slowly paralyzed as

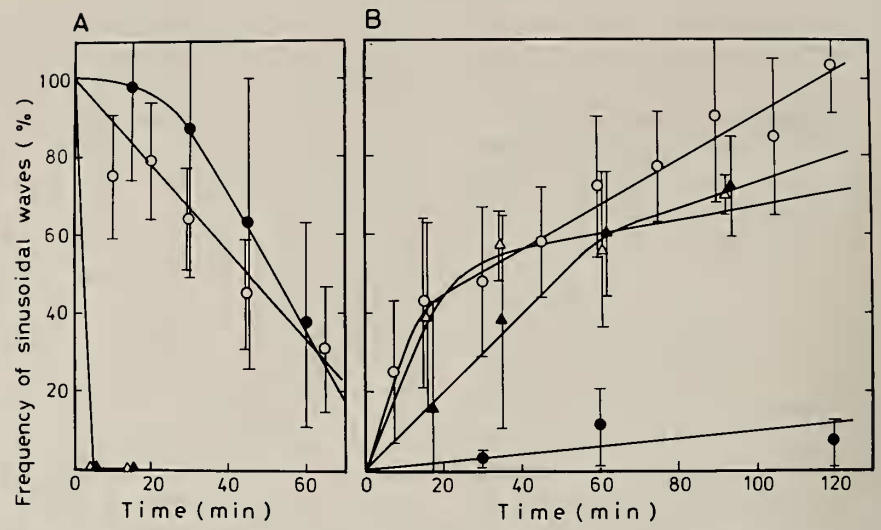


FIG. 5. Paralysis by trichlorfon and recovery. (A) Newly hatched larvae were exposed to 1 mM trichlorfon and changes in the sinusoidal frequency were followed. (B) Recovery from the paralysis was followed after an extensive wash of the completely paralyzed worms. The sinusoidal activity of each strain means a relative value to that prior to trichlorfon exposure. The mean values of seven worms are presented with bar for standard deviation. Wild type (○), *ace-2* (*g72*) *ace-3* (*dc2*) (△), *ace-3* (*dc2*) *ace-1* (*p1000*) (▲), and *ace-1* (*p1000*) *ace-2* (*g72*) (●).

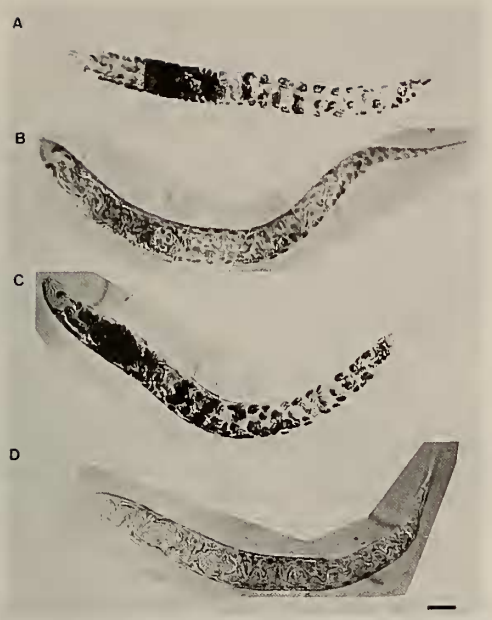
were wild-type animals, but the *ace-3* defective mutants were instantaneously paralyzed (Fig. 5A). Animals preserving either the *ace-1* or *ace-2* gene alone recovered from the trichlorfon-induced paralysis in the same manner as wild-type animals, though a portion of the population did not. On the other hand, animals retaining only the *ace-3* gene had difficulty recovering from paralysis (Fig. 5B). It is likely that the higher concentration of trichlorfon induced a slow but irreversible inactivation of class C AChE, whereas the inactivation of class A and B AChE is completely reversible.

Effects of trichlorfon on the localization of AChE staining

Since trichlorfon principally inhibits AChE activity, the pattern of the AChE staining will diversely change during the paralytic and recovery states after trichlorfon exposure. As described above, newly hatched larvae of wild-type and double mutants of the *ace* genes showed characteristic AChE staining (Fig. 6 A-D). However, the staining was completely lost in any strain during paralysis as shown in the wild-type animal (Fig. 6E). Staining along the ventral nerve cord recovered partially accompanying recovery from the paralysis, but staining at the head region did not recover (Fig. 6F-I). Thus movement recovery does not need complete restoration of the AChE staining.

Effects of trichlorfon treatment on ACh levels

Changes in ACh levels after trichlorfon exposure were also studied (Fig. 7). To prepare animals in the same state of paralysis, each strain was incubated at different concentrations of trichlorfon. At the paralyzed state, the ACh levels were slightly elevated in the *ace-1 ace-3* and *ace-3 ace-2* mutants as seen in the wild type, but greatly elevated in the *ace-1 ace-2* mutant. Therefore, the extent of the paralysis did not apparently correlate with the accumulation of ACh. However, since the ACh level was assayed after washing the animals to remove trichlorfon, it is conceivable that the difference in ACh levels among respective mutants reflects the difference in reversibility of the inactivated enzyme.



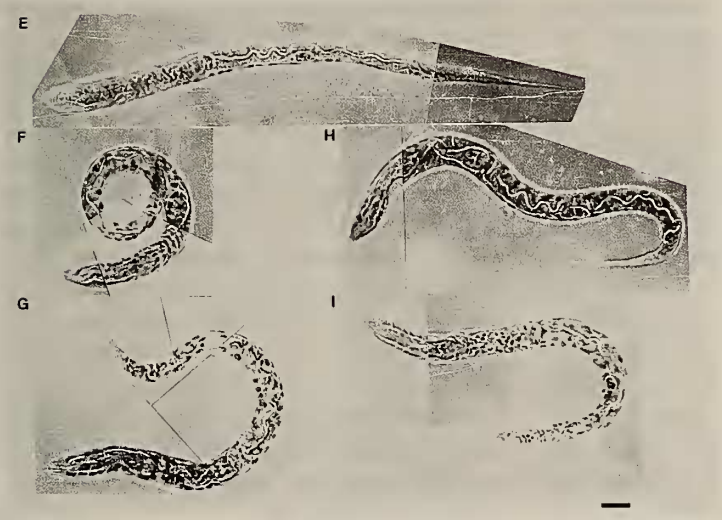


FIG. 6. Changes in AChE staining caused by trichlorfon. Newly hatched larvae became paralyzed, then recovered as in Figure 5. Animals before trichlorfon treatment (A-D). A completely paralyzed wild type (E). Animals recovered from the paralysis (F-I). Wild type (A, E, F), *ace-2* (*g72*) *ace-3* (*dc2*) (B, G), *ace-3* (*dc2*) *ace-1* (*p1000*) (C, H), and *ace-1* (*p1000*) *ace-2* (*g72*) (D, I). Animals are oriented anterior side to the left and the ventral side on bottom. Bar =200 μm.

DISCUSSION

To understand the roles that the three classes of AChE may play, we studied the enzyme activity, histochemistry and ACh levels in mutant and wild-type *C. elegans*. Classes A and B AChE have several similar properties. That is, the developmental pattern of the expression of the two enzymes is comparable (Fig. 1) and the inhibitory pattern by trichlorfon is similar (Fig. 4), although both enzymes seem to functionally overlap but are not coincident. For example, mutants retaining class A AChE are more sensitive to trichlorfon than those containing only class B AChE. The staining pattern of AChE of whole mutant animals containing only class B was similar as that of the wild type. However, the staining of animals containing only class A AChE is faint in the body region. Classes A and B AChE differ in Km values [9, 12] and the extent of membrane association [9]. Solubilized class A AChE is as inhibited by trichlorfon as the crude enzyme but solubilized class

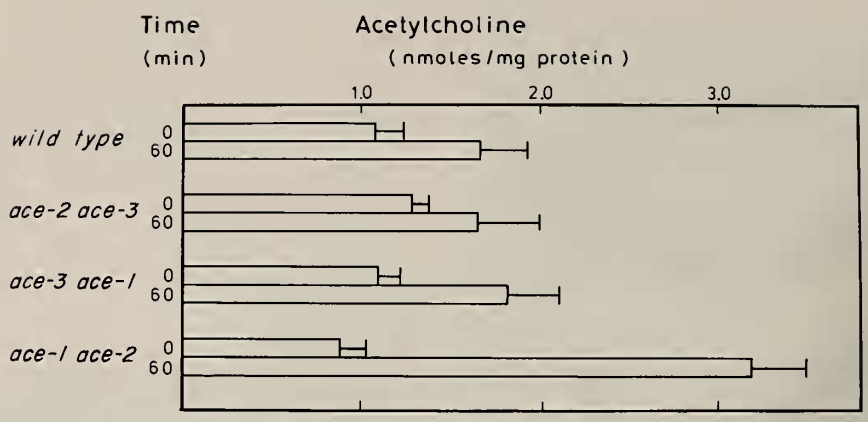


FIG. 7. Changes in ACh levels by trichlorfon exposure. The newly hatched larvae of the wild type and the the *ace-1 ace-2* mutant were exposed to 1 mM, and the *ace-2 ace-3* and the *ace-3 ace-1* mutants to 0.1 mM trichlorfon, respectively. Under these conditions, four strains reached in approximately the same paralyzed state. At 60 min after exposure, animals were collected for the assay of ACh levels. Standard deviations of the mean values of three assays are indicated by vertical bars.

AChE is less inhibited (unpublished results).

Class C AChE was found to be greatly different from classes A and B AChE in expression, distribution and response to trichlorfon. The class C enzyme is resistant to trichlorfon but the strain retaining only the enzyme is hardly recovered from the trichlorfon-induced paralysis. The activity of class C AChE is one-fiftieth lower than the activity of A and B AChE, peaks at the early L4 stage, and distributed in the body rather than in the head region.

For normal locomotion of *C. elegans,* it is essential that either the *ace-1* or the *ace-2* gene is intact, suggesting that a decrease in the total AChE activity rather than in the specific AChE, leads to locomotive abnormalities. In other words, the reduction of ACh hydrolysis and therefore, the accumulation of ACh, results in the movement abnormality. However, the extent of the elevation of the total ACh levels and the movement abnormality is not always correlated because, in the *ace-1 ace-2* mutant, the movement is abnormal throughout development, but the ACh levels are normal at the early larval stage. Also, under the trichlorfon-induced paralyzed states with wild-type animals and *ace* double mutants, the extent of the ACh elevation of the *ace-1 ace-2* mutant greatly differs from those remaining. However, the rule conforms at the adult stage but not at the early larval stage, because, in the *ace-1 ace-2* mutant, ACh levels are normal at the L1 and L2 stage, but are elevated at the adult stage.

The present findings can not fully explain why three types of AChE are present in *C. elegans.* We showed that slight elevation of ACh results in a movement abnormality, indicating that the consequent accumulation of ACh levels leads to lethal over-stimulation of some control and peripheral neurons. The presence of heterogeneous types of AChE, whose localization, expression and abundance differ, is useful for the prompt removal of stagnated ACh. Animals containing only class B AChE are indistinguishable from the wild type in the distribution and the staining intensity. Class A AChE normally presents at the anterior region of the body but is less abundant at the posterior region. Although class C AChE is much less abundant, it is detectable at the posterior region of the body. Therefore, class B AChE may play a major role and class A and C AChE a regionally supplementary role in the hydrolysis of ACh. Thus, the presence of different types of AChE may be useful for regulating the local concentration of ACh in the synapse. The electrical organ of *Torpedo californica* has also structurally distinct molecular forms of AChE [1]. They are differently distributed in the electric organ and suggested that they may play different roles on the hydrolysis of

ACh.

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